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Biocompatibility Evaluation of Ionic- and Photo-Crosslinked Methacrylated Gellan Gum Hydrogels: In Vitro and In Vivo Study

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In this study, the stability and biocompatibility of methacrylated gellan gum hydrogels, obtained either by ionic- (iGG-MA) or photo-crosslinking (phGG-MA), were evaluated in vitro and in vivo. Size exclusion chromatography analysis of the methacrylated gellan gum (GG-MA) powder revealed that molecular weight is lower as compared to the non-modified material, i.e., low acyl gellan gum. The water uptake and swelling of iGG-MA and phGG-MA hydrogels were investigated in phosphate-buffered saline solution (pH 7.4). The biocompatibility of the hydrogels was firstly evaluated by producing cell-laden hydrogels. The in vitro cells encapsulation study showed that lung fibroblast cells (L929 cells) and human intervertebral disc (hIVD) cells are viable when cultured within both hydrogels, up to 21 days of culturing. The iGG-MA and phGG-MA hydrogels were also subcutaneously implanted in Lewis rats for 10 and 18 days. Tissue response to the hydrogels implantation was determined by histological analysis (haematoxylin-eosin staining). A thin fibrous capsule was observed around the implanted hydrogels. No necrosis, calcification, and acute inflammatory reaction were observed. The results presented in this study demonstrate that iGG-MA and phGG-MA hydrogels are stable in vitro and in vivo, support L929 and hIVD cells' encapsulation and viability, and were found to be well-tolerated and non-toxic in vivo.

1. Introduction

An intervertebral disc (IVD) is constituted by two structures, an outer multilayer fiber structure (annulus fibrosus, AF) and a gel-like inner core (nucleus pulposus, NP), which are sandwiched in part between two cartilage endplates.[1] In a diseased IVD, significant alterations in disc occurs, namely: (i) biochemical composition changes, i.e., extracellular matrix (ECM) remodelling, (ii) loss of hydration of NP, (iii) decrease in disc height, (iv) deterioration of facet cartilage, and (v) disc protrusion or herniation.[2–4] Conservative approaches for treatment of degenerating IVD are mainly based on drug administration,[5] but severe disc problems often require surgical intervention such as discectomy, spine fusion, and disc replacement.[6–8]

Pre-clinical research efforts are focused on developing new strategies to decrease progression and prevent some of the changes observed in a degenerating IVD.[9,10] With the advent of tissue engineering, the use of stem cells and platelet-rich plasma (PRP)-based strategies comprise new regenerative possibilities for treating IVD degeneration.[11–13] However, the use of cells or PRPs alone may not be feasible, mainly due to the short half-life of the GFs and the need to comply with fully restoration of IVD functionality (e.g., disc height).[6]

Hydrogels have attracted a great deal of attention as potential NP substitutes, because they allow irregular surgical defects to be completely filled using minimally invasive techniques. In addition, hydrogels also present the advantage of functionally mimicking the native ECM, supporting NP cells functions and

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sustaining the release of bioactive molecules.[14–18] Reza et al.[19] proposed the photo-crosslinked carboxymethyl-cellulose hydrogels as tunable biomaterials for NP cell encapsulation, although these lack the adequate mechanical properties. By its turn, Su et al.[20] reported on oxidized hyaluronic acid/adipic acid dihydrazide (oxi-HA/ADH) hydrogel formulations for NP regeneration. The oxi-HA/ADH hydrogels changed from liquid form into a gel-like structure within few minutes, but low cellular viability was observed for certain oxi-HA/ADH hydrogel mixtures, in vitro. Moreover, the fast degradation of the hydrogels and poor long-term stability can compromise future application in NP tissue engineering strategies.

Our group[21,22] is a pioneer in processing hydrogels based on gellan gum (GG), which is a polysaccharide obtained from *Sphingomonas elodea*, for several tissue engineering applications. The GG hydrogel gelation occurs by ionic-crosslinking with divalent cations. The GG hydrogel has been shown to efficiently sustain the deliver and growth of human articular chondrocytes and support the deposition of a hyaline-like ECM, leading to the formation of a functional cartilage.[23] Although GG produces stable gels, the mechanical functionality and long-term stability of GG needs to be tuned, in order to fully comply with the IVD requirements, namely to be used as acellular and cellular NP substitutes. Recently, methacrylated gellan gum (GG-MA) hydrogels, the so-called ionic- (iGG-MA) and photo-crosslinked (phGG-MA) methacrylated gellan gum, were produced through chemical modification by means of methacrylation of low acyl GG.[24,25] The main advantages of the GG-MA hydrogels as compared to others[19,20,22,25] are: (i) the possibility to control endothelial cells infiltration and blood vessel ingrowth's,[26] (ii) tunable and improved mechanical properties, and (iii) *in situ* gelation, within seconds to few minutes.[24,25] In previous work,[25] the modified material and the developed iGG-MA and phGG-MA hydrogels were extensively characterized in respect to its physicochemical properties. In particular, the mechanical behaviour of the iGG-MA and phGG-MA hydrogel discs was characterized by dynamic mechanical analysis (DMA). The storage modulus presented by the phGG-MA discs at 1 Hz was higher (122.8 ± 8.3 kPa) than that of GG hydrogels (56.2 ± 1.4 kPa), while in the iGG-MA hydrogel discs an intermediate behaviour (89.5 ± 7.4 kPa) was observed. By performing GG methacrylation with different reaction conditions and using different crosslinking methods for hydrogel formation, it is possible to tune the crosslinking degree of the polymer, which then influences its mechanical properties, degradation profile and swelling capacity. Moreover, a more compact microstructure, as observed in iGG-MA and phGG-MA as compared to GG hydrogels, has been shown to be more effective in preventing infiltration of endothelial cells and blood vessels.[26] Therefore, this strategy can be used for tailoring the final properties of the GG-based hydrogels, in order to mimic the functional properties of the tissue aimed to regenerate. Also in this work,[25] the authors demonstrated, by following standard tests for assessment of biocompatibility safety of medical devices, that the degree of methacrylation of the modified material (i.e., GG-MA) was not cytotoxic to cells. Despite the promising physicochemical properties of the iGG-MA and phGG-MA hydrogels and non-cytotoxicity, no information on the cells encapsulation ability and in vivo biocompatibility of the hydrogels has been reported.

In this work, our aim was to investigate the long-term in vitro stability and screen the biocompatibility of the iGG-MA and phGG-MA hydrogels, in vitro and in vivo. Moreover, the molecular weight distribution of the GG-MA was determined by size exclusion chromatography. Degradation and water uptake studies were performed by means of soaking the iGG-MA and phGG-MA hydrogels in a phosphate buffered saline solution (pH 7.4, 37 °C) for times ranging from 1 up to 90 days. The ability of the iGG-MA and phGG-MA hydrogels for cells encapsulation was evaluated by culturing the well-established immortalized mouse lung fibroblast cell line and primary human IVD cells within the hydrogels, for 1 up to 21 days. After each culturing time point, calcein-AM staining was performed to qualitatively evaluate the cells viability. For the purpose of the in vivo study, subcutaneous implantation of the iGG-MA and phGG-MA hydrogels in a rat model was performed. After 10 and 18 days of implantation, the haematoxylin and eosin (H&E) stained sections of explants were observed for evaluation of biocompatibility and cell infiltration into the hydrogels implants.

2. Results

2.1. Characterization of the Methacrylated Gellan Gum Hydrogels

To further investigate the chemical modification of low acyl gellan gum (GG) with glycidyl methacrylate (GMA), the molecular weight distribution of resulting methacrylated gellan gum (GG-MA) powder was determined by size exclusion chromatography (SEC). **Table 1** shows that the molecular weight (Mw) of GG-MA is slightly lower as compared to GG. Additionally, the calculation of the polydispersity index (Mw/Mn) reveals that both polymers present a uniform distribution of molecular weight.

The long-term stability of the ionic- (iGG-MA) and photo-crosslinked (phGG-MA) methacrylated gellan gum hydrogels was evaluated by soaking the hydrogels in a PBS solution (pH 7.4 at 37 °C) up to 90 days. **Figure 1** shows the weight loss and water uptake profile of iGG-MA and phGG-MA hydrogels. No significant weight loss was observed among the hydrogels until 90 days (Figure 1A). Despite it was observed an increase in weight loss of iGG-MA hydrogels after 90 days, as compared to phGG-MA hydrogels, this value is not statistically significant ($p > 0.05$). Regarding the water uptake ability of the hydrogels, although the results suggest that the iGG-MA hydrogels were able to retain more water as compared to phGG-MA hydrogels until 60 days (Figure 1B), statistical analysis has shown that this difference is not significant. Additionally, the higher water content shown by the phGG-MA hydrogels as compared to iGG-MA hydrogels after 90 days is not statistically significant ($p > 0.05$).

2.2. Cell-Laden Hydrogels

In order to evaluate the biocompatibility of iGG-MA and phGG-MA hydrogels in vitro, different cell types were encapsulated

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Table 1. Molecular weight determination of gellan gum (GG) and methacrylated gellan gum (GG-MA) measured by size exclusion chromatography.

Sample	Mw [kDa]	Mn [kDa]	Polydispersity index [Mw/Mn]
GG	71.1	63.9	1.11
GG-MA	70.6	62.4	1.13

within the hydrogels and cultured up to 21 days. Cells viability was visualized by means of staining the cell-laden iGG-MA and phGG-MA hydrogels with calcein-AM after each culturing time. **Figure 2** shows that mouse lung fibroblast (L929) cells remained viable until 21 days of culturing, as observed by the green fluorescence, and where generally well dispersed within the matrices of both hydrogels. **Figure 3** also revealed that the hydrogels can support the viability of primary human IVD (hIVD) cells. Human IVD cells were also homogeneously distributed within the iGG-MA and phGG-MA hydrogels. Moreover, it was observed that the hIVD cells maintained its round-shape morphology during culturing time.

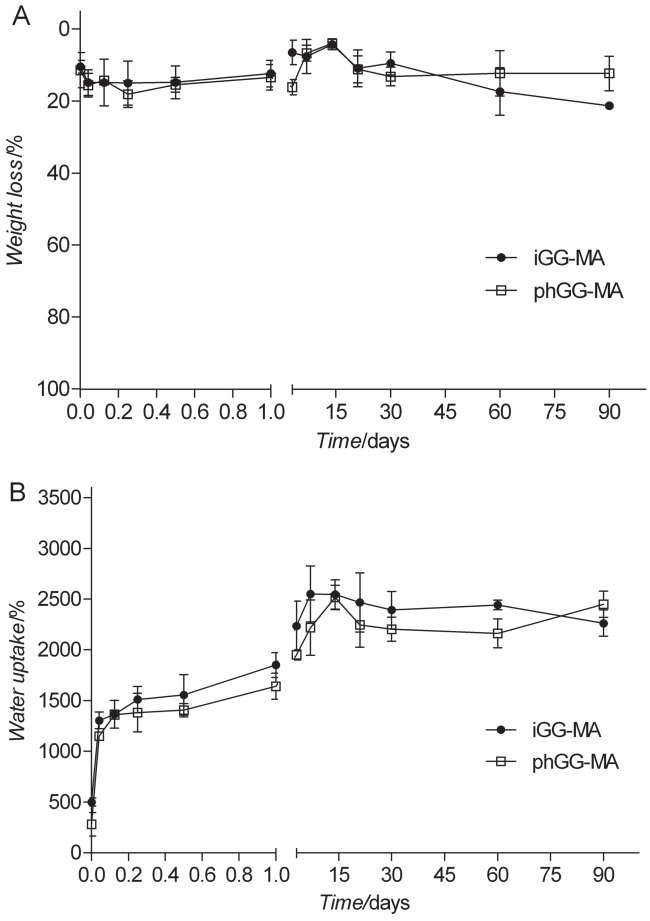

Figure 1. Weight loss (A) and water uptake (B) of ionic- (iGG-MA) and photo-crosslinked (phGG-MA) methacrylated gellan gum hydrogel discs. All samples were immersed in PBS (pH 7.4; 37 °C and 60 rpm) for times ranging from 1 up to 90 days. Values are expressed as mean ± SD (n = 9).

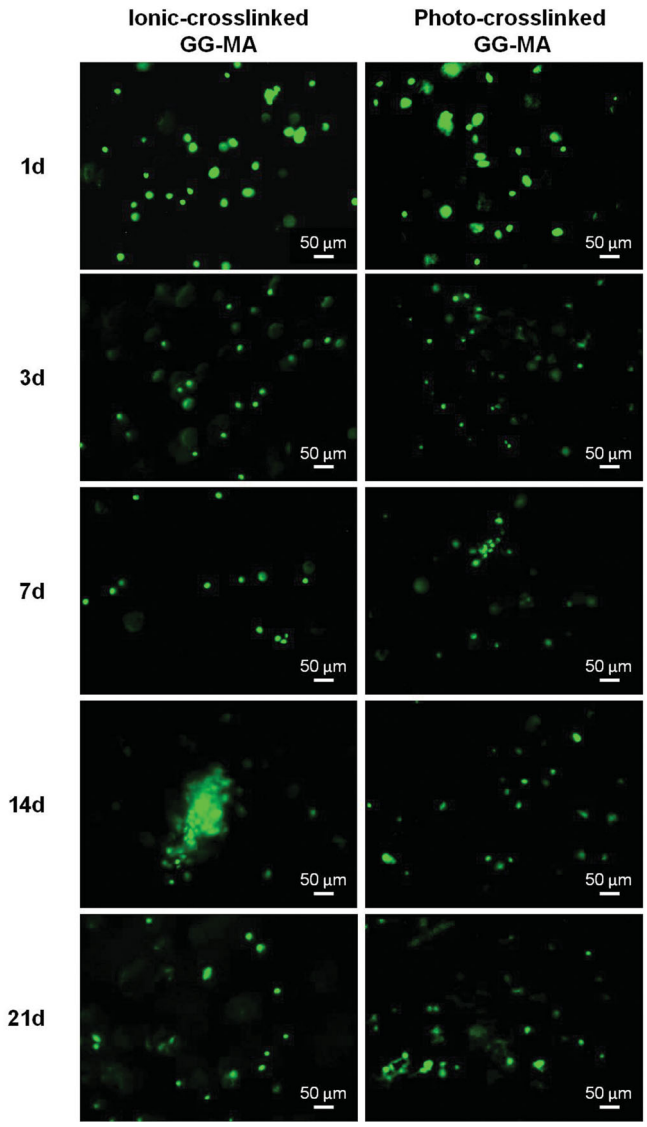

Figure 2. Fluorescence microscopy images of the L929 cells encapsulated within the ionic- and photo-crosslinked methacrylated gellan gum (GG-MA) hydrogels and cultured from 1 up to 21 days. The cells were stained with calcein-AM (green fluorescence), prior to observation.

2.3. In Vivo Biocompatibility Assessment

After subcutaneous implantation in a rat model, the iGG-MA and phGG-MA implants were retrieved and processed for histological analysis. No signs of infection, necrosis or calcification were detected at the implantation sites, for all implants after 10 and 18 days of implantation. **Table 2** summarizes the semi-quantitative analysis of the inflammatory cells within the iGG-MA and phGG-MA hydrogel explants. At day 10, both materials showed a moderate infiltration of granulocytes and macrophages. It was also observed a minor number of non-phagocytic cells and a moderate amount of fibroblasts around the hydrogel implants. At day 18, scaffolds showed a minor infiltration of granulocytes, macrophages and non-phagocytic

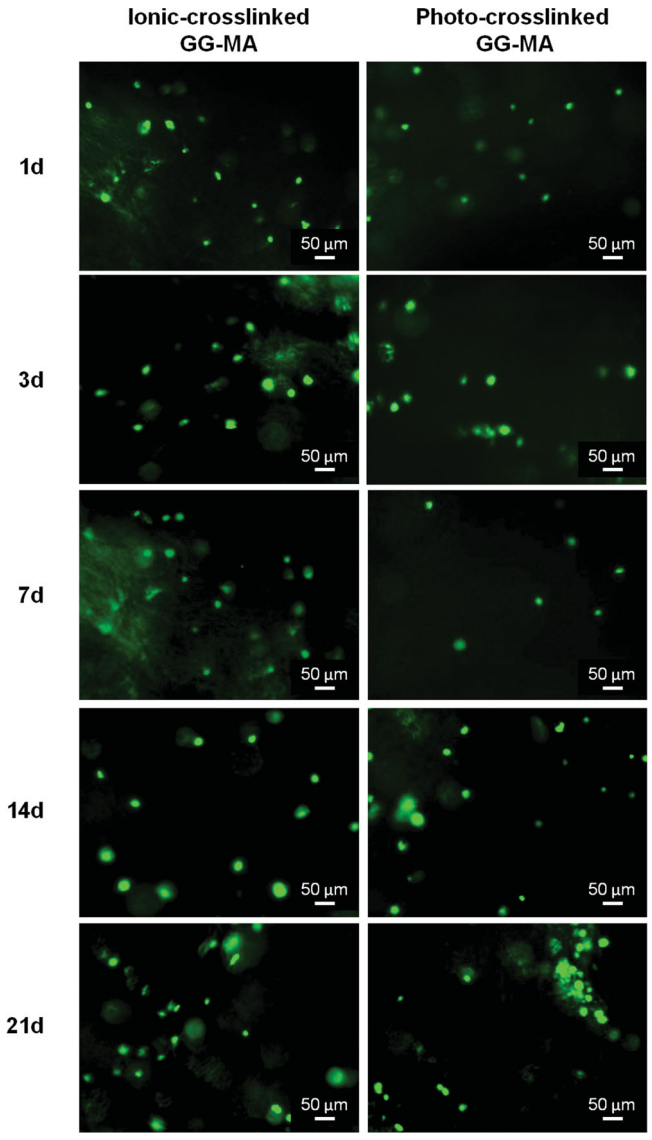

Figure 3. Fluorescence microscopy images of the hIVD cells encapsulated within the ionic- and photo-crosslinked methacrylated gellan gum (GG-MA) hydrogels and cultured from 1 up to 21 days. The cells were stained with calcein-AM (green fluorescence), prior to observation.

cells. A small infiltration of fibroblasts was also detected. The absolute numbers of polymorphic nuclear cells (PMNs) and non-phagocytic cells are lower as compared to phagocytic cells.

Figure 4 shows the light microscopy photographs of the the iGG-MA and phGG-MA explants after 10 days of subcutaneous implantation, which were stained with haematoxylin & eosin (H&E). A connective thin fibrous tissue was observed surrounding both implanted iGG-MA and phGG-MA hydrogels, which assumed the form of fibres. The central part presented void spaces and no cells were observed within both iGG-MA and phGG-MA hydrogels. At high magnification, H&E images clearly showed newly formed connective tissue septa surrounding the periphery of the hydrogels (black arrows). No inflammatory cells such as macrophages and foreign body giant cells were observed. Similar observations for both hydrogels after 18 days of implantation were found, i.e., no inflammatory cells were present in the implants (**Figure 5**).

Table 2. Cellular response to ionic- (iGG-MA) and photo-crosslinked (phGG-MA) hydrogel implants. Cells were scored from absent (–) to abundantly present (+++).

Implantation time [days]	Implant material	PMNs[a)]	Phagocytic cells[b)]	Non-phagocytic cells[c)]	Fibroblasts
10	iGG-MA	++	++	+	++
	phGG-MA	++	++	+	++
18	iGG-MA	+	+	+	+
	phGG-MA	+	+	+	+

[a)]PMNs = polymorphic nuclear cells, i.e., granulocytes; [b)]Phagocytic cells include macrophages and monocyte-derived giant cells; [c)]Non-phagocytic cells include lymphocytes, plasma cells and mast cells.

3. Discussion

Photo-polymerization of hydrogels is being investigated due to the possibility of tailoring the physicochemical properties of biomaterials namely, degradation or resorption rate, swelling, permeability to cells/molecules, and mechanical properties.[19,27,28] As aforementioned, our group has been proposing the use of ionic-crosslinked low acyl gellan gum (GG) hydrogels for tissue engineering scaffolding applications. Photo-polymerization may be used as a reliable strategy for tuning the properties of materials. Thus, we were encouraged to chemically modify the low acyl GG with glycidyl methacrylate in order to develop a photo-polymerizable hydrogel that can best resemble the physical characteristics of NP tissue. Previously, we have shown that photo- (phGG-MA) and ionic-crosslinked (iGG-MA) methacrylated gellan gum hydrogels present a storage modulus at 1 Hz of 122.8 ± 8.3 kPa and 89.5 ± 7.4 kPa, respectively.[25] The GG-MA hydrogels present improved mechanical performance as compared to that of GG hydrogels (56.2 ± 1.4 kPa) which results from a higher crosslinking density. In fact, rheological analysis has been shown that native human NP presents a shear modulus of 7–20 kPa that corresponds to a Young's modulus of 20–60 kPa.[29,30] These values, which are similar to those obtained for GG hydrogels, were however obtained *post mortem* and they may not translate the real native NP properties.[31] The sheep model is often used to perform IVD mechanical studies since it is believed to be one of the animal models that best resemble the human IVD.[32] Dynamic compression studies revealed that NP sheep has a storage modulus of 64 ± 28 kPa,[29] which is lower as compared to the phGG-MA and iGG-MA hydrogels.

In this study, SEC analysis revealed that GG-MA has a molecular weight slightly lower than GG. From results, we can state that the methacrylation of GG did not significantly change its molecular weight and polydispersity index. It should be highlighted that methacrylation of GG improved the water-solubility of the polymer, and contrarily to GG, homogeneous dissolution of GG-MA powders can be performed at room temperature.

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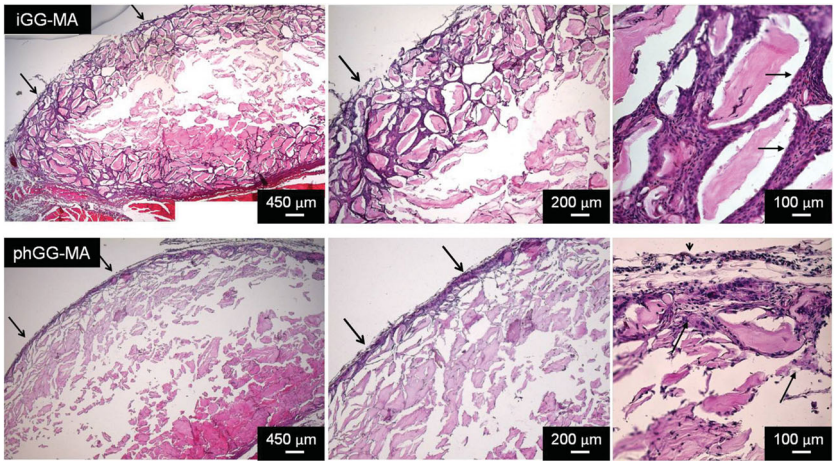

Figure 4. Light microscopy images of ionic- (iGG-MA) and photo-crosslinked (phGG-MA) methacrylated gellan gum hydrogel explants sections stained with H&E, after 10 days of subcutaneous implantation. Arrows indicate the newly formed connective tissue septa surrounding the periphery of the hydrogels.

Following the initial study[25] on the gellan gum-based hydrogels stability, the iGG-MA and phGG-MA hydrogels were selected as the most promising biomaterials for NP regeneration. The long-term stability studies of the iGG-MA and phGG-MA hydrogels showed that no significant weight loss was observed among the hydrogels until 60 days, but a slight increase in weight loss of iGG-MA hydrogels as compared to phGG-MA hydrogels, although not statistically significant, was observed after 90 days. The water uptake study revealed that the phGG-MA hydrogels were able to swell less as compared to iGG-MA hydrogels until 60 days of soaking. Although the differences were not statistically significant, these results can be attributed to a higher crosslinking density of the phGG-MA hydrogels, consistent with a tighter matrix which thus is less able to retain water. After 90 days, the phGG-MA hydrogels presented higher water content as compared to that of iGG-MA hydrogels, again not statistically significant, which can be attributed to the increase in the weight loss of iGG-MA hydrogels. This result is particularly interesting since we are able to tune both hydrogels' water retention and degradation rate by means of playing with the method of crosslinking of the GG-MA biomaterial. Actually, the developed iGG-MA and phGG-MA hydrogels presented a better long-term stability as compared to other reported biomaterials[20] with promising application in NP tissue engineering. Degradation and swelling assays need to be performed for longer periods of incubation, as well as in enzymatic medium, in order to better characterize the in vitro stability of the iGG-MA and phGG-MA hydrogels.

Tissue engineering of degenerated NP demands for a hydrogel substitute that can mimic its native avascular structure. Thus, non- or anti-angiogenic hydrogels are needed for this particular application, since angiogenesis is also one of the factors promoting IVD degeneration and pain.[33] In previous work,[26] the angiogenic response of iGG-MA and phGG-MA hydrogels was investigated by performing the chorioallantoic membrane (CAM) assay, a well-established model to investigate angiogenesis in vivo.[34,35] Results have shown that the iGG-MA and phGG-MA hydrogels were well tolerated, adhered firmly to the CAM and did not elicit any inflammatory reaction. More importantly, the iGG-MA and phGG-MA hydrogels were able to control endothelial cells infiltration and blood vessels invasion during the period of implantation, properties that are a must when designing an adequate NP substitute.

Photo-polymerized hydrogels are also appealing from both surgical and tissue engineering point of view, as it can be applied at defect area using minimally invasive procedures, while simultaneously enable cells encapsulation and *in situ* gelation to occur.[27] But, photo-polymerization frequently involves use of photo-initiators, UV light, and increase in gels temperature during the gelation process, and thus the developed hydrogels cannot be utilized in tissue engineering because of the lack of cyto- and bio-compatibility.

The in vitro and in vivo biocompatibility of the developed iGG-MA and phGG-MA hydrogels was evaluated following well-established protocols.[36–38] In the current work, we have firstly investigated the ability of the GG-MA biomaterial towards cells encapsulation using two distinct processes, i.e., ionic- and photo-crosslinking. Both methods allowed us to homogeneously seed the L929 cells and hIVD cells throughout the gels and to form stable hydrogels within seconds to few minutes. The viability assay showed that iGG-MA and phGG-MA hydrogels supported cells functions up to 21 days of culturing. Thus,

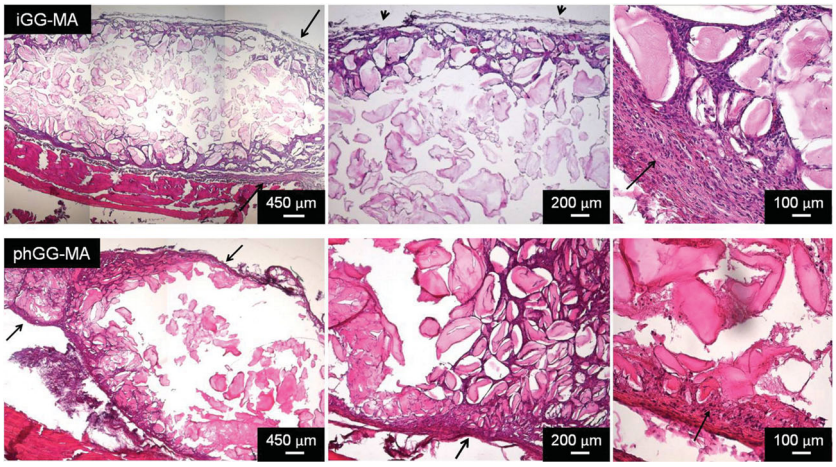

Figure 5. Light microscopy images of ionic- (iGG-MA) and photo-crosslinked (phGG-MA) methacrylated gellan gum hydrogel explants sections stained with H&E, after 18 days of subcutaneous implantation. Arrows point to the newly formed connective tissue septa surrounding the periphery of the hydrogels.

the present data showed that the use of MBF photo-initiator and photo-polymerization conditions, i.e., UV light (366 nm) and gelation time (6 minutes) did not affect cells viability. It has been reported that potential cellular damage can occur due to UV radiation, but it is dependent on cell type, time of exposure and type/concentration of the photo-initiator.[39–41] In future work, we will investigate possible cellular DNA damage, in short- and long-term, after exposure to UV light in these conditions. In order to investigate the cellular response to the iGG-MA and phGG-MA hydrogels after subcutaneous implantation for 10 and 18 days, masked microscopic examinations were performed by means of H&E staining for inflammatory cells.[42] After 10 days of implantation, both implant materials showed a moderate infiltration of granulocytes and macrophages, minor of non-phagocytic cells and a moderate amount of fibroblasts around the hydrogel implants. By its turn, no infiltration of granulocytes, macrophages and non-phagocytic cells was observed after 18 days of implantation. Thus, the in vitro and in vivo data demonstrated that both hydrogels present a good tissue compatibility and non-toxicity.

Although this study was focused on screening the long-term stability, and in vitro and in vivo biocompatibility of both iGG-MA and phGG-MA hydrogels, future work will investigate the effect of environmental stimuli (e.g., growth factor supplementation - TGF-beta 3), on maintenance of phenotypic ECM components in cell-laden hydrogels. In addition, long-term in vivo experiments need to be performed in order to further determine degradability rate of the developed hydrogels.

4. Conclusions

In this work, we demonstrate that photo-polymerization of methacrylated gellan gum (GG-MA) allowed improvement of both the stability of the hydrogels and to tune swelling. Moreover, the ionic- (iGG-MA) and photo-crosslinked (phGG-MA) hydrogels were shown to be effective on supporting cells' encapsulation and viability up to 21 days of culturing. The in vivo biocompatibility screening showed that iGG-MA and phGG-MA hydrogels do not elicit any deleterious effect or inflammatory reaction. Thus, this study reveals that the well-tolerated and non-toxic ionic- and photo-crosslinked methacrylated gellan gum hydrogels possess promising physicochemical and biological properties for finding application as viable NP substitutes.

5. Experimental Section

Materials: Methacrylated gellan gum (GG-MA) powder was produced by means of chemical modification of low-acyl gellan gum (LA-GG, Gelzan CM; Sigma-Aldrich Co., USA). Glycidyl methacrylate (GMA, 97%) and methyl benzoylformate photo-initiator (MBF, 98%) were purchased from Sigma-Aldrich Co. (USA). All reagents were obtained from Sigma-Aldrich Co. (USA), unless otherwise indicated.

Synthesis of GG-MA Powders and Preparation of the Ionic- and Photo-Crosslinked GG-MA Hydrogel Discs: The methacrylation of gellan gum was performed by reaction of LA-GG with GMA at 20-fold molar excess in respect to the repeating unit of LA-GG, as previously described by Silva-Correia et al.[25] Briefly, a solution of LA-GG at 1% (w/v) final concentration was prepared in distilled water at room temperature and under vigorous agitation. The LA-GG solution was heated to 90 °C in a water bath in order to obtain a homogeneous dispersion. The LA-GG solution was allowed to cool down until reaching room temperature and an appropriate amount of GMA was added. The pH of the reaction mixture was automatically adjusted to 8.5 with 1 M sodium hydroxide (NaOH; Panreac Química SAU, Spain) by means of using an automatic pump (Fusion 200; Chemyx Inc., USA). Then, the GG-MA was precipitated with cold acetone (0.5 volumes; Panreac Química SAU, Spain) and the excess of GMA and acetone eliminated off by dialysis (cellulose membrane, MW cut-off 12 kDa; Sigma, USA) for 7 days. Finally, the purified GG-MA dialyzed solution was frozen at −80 °C and the GG-MA powders were obtained by freeze-drying (Cryodos −80; Telstar, Spain).

The ionic- (iGG-MA) and photo-crosslinked (phGG-MA) methacrylated gellan gum hydrogel discs, possessing a diameter of 7 mm and height of approximately 4 mm, were obtained by dissolving the GG-MA powder in distilled water at a final concentration of 2% (w/v), under vigorous agitation and at room temperature. For the preparation of iGG-MA hydrogel discs, 150 µL of the 2% (w/v) GG-MA aqueous solution were transferred to a silicone mould and gelation of the gels was induced by immersion in PBS (pH 7.4) for 30 minutes. The phGG-MA hydrogel discs were obtained by means of adding 0.1% (w/v) photo-initiator MBF to the 2% (w/v) GG-MA aqueous solution. Then, 150 µL of the mixture were transferred to a silicone mould and the hydrogel discs were obtained by exposing the gels to ultraviolet light (366 nm, UV lamp Triwood 6/36; Bresciani srl., Italy) for the period of 6 minutes. Finally, the phGG-MA hydrogel discs were further equilibrated in PBS (pH 7.4) for 30 minutes.

For the purpose of the in vitro cell encapsulation and in vivo studies, GG-MA powders were sterilized under an ethylene oxide gas atmosphere, and the iGG-MA and phGG-MA hydrogel discs were prepared in sterile conditions, i.e., using sterile materials and reagents inside a laminar-flow hood (BH-EN 2004-D; Faster, UK).

Molecular Weight Determination: Weight-average molecular weights (M_W) and number-average molecular weights (M_n) were determined by size exclusion chromatography (SEC) with triple detection (Viscotek Triple Detector Array–TDA 305; Malvern, UK), using OmniSec software (Viscotek, version 4.6.1.354; Malvern, UK). A Viscotek VE 1122 solvent delivery system was connected to one Viscotek AGuard 50 × 6.0 mm pre-column, one Viscotek A2000 column and one Viscotek A2500 column (300 × 7.8 mm). A Viscotek TDA 305 triple detector array was used, including right-angle light scattering (RALS), refractive index (RI) and Viscosimetry detectors. A solution of NaN_3 at 0.02% (w/v) was used as eluent and the samples were eluted at a flow rate of 1 mL·min^{-1} at 60°C. Polymer concentration was 1 g·L^{-1} and polymer solutions were filtered through 0.45 µm pore size membranes before injection.

Water Uptake and Weight loss Studies: The iGG-MA and phGG-MA hydrogels discs swelling ability and weight loss were investigated by means of soaking the hydrogel discs in PBS (pH 7.4), from 1 up to 90 days. The study was carried out at 37 °C and under constant agitation (60 rpm). After each time point, the water uptake and weight loss of the hydrogels were calculated as previously described elsewhere.[25] The studies were performed using a minimum of three samples per experimental condition ($n = 9$) and values are expressed as mean ± SD. Statistical analysis (GraphPad Prism; GraphPad Software, San Diego, USA) was performed using a Two-way analysis of variance followed by Bonferroni *post*-test with a minimum confidence level of 0.05 for statistical significance.

In Vitro Cells Encapsulation Study: L929 Cells Encapsulation and Culturing: The ability of ionic- and photo-crosslinked GG-MA hydrogels for encapsulating cells in mild conditions (room temperature) was evaluated using the well-established immortalized mouse lung fibroblast cell line (L929 cells), purchased from European Collection of Cell Cultures (ECACC, UK). L929 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) supplemented with 10% foetal bovine serum (FBS; Biochrom, Germany) and 1% of an antibiotic-antimycotic mixture (Invitrogen, USA) containing 10 000 units·mL^{-1} penicillin G sodium, 10 000 µg·mL^{-1} streptomycin sulphate and 25 µg·mL^{-1} amphotericin B as Fungizone Antimycotic in 0.85% saline.

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The L929 cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2, and the medium changed every two days. A chemical method using trypsin (0.25% trypsin/EDTA solution, Sigma, USA) was used to promote cell detachment from confluent culture flasks. Afterwards, a diluted cell suspension was prepared and centrifuged at 1200 rpm for 5 minutes. The medium was completely aspirated and the cell pellet was re-suspended in the respective hydrogel solution to yield a final concentration of 2.5×10^6 cells·mL^{-1}. Solutions at 2% (w/v) made of GG-MA and GG-MA with 0.1% (w/v) MBF were prepared under sterile conditions, prior cells encapsulation. Cell-laden gels were centrifuged at 600 rpm for 3 minutes to allow homogenization of cells. Then, L929 cells-laden hydrogels (200 µL/disc) were transferred to silicone moulds to produce discs with a diameter of 7 mm. For obtaining cell-laden iGG-MA hydrogels, the iGG-MA gel with encapsulated L929 cells (5×10^5 cells/disc) was then immersed in PBS solution for crosslinking. For producing cell-laden phGG-MA hydrogels, the GG-MA gels containing MBF at 0.1% (w/v) with L929 cells were exposed to UV light for 6 minutes (366 nm; UV lamp Triwood 6/36, Bresciani srl., Italy), and then equilibrated in PBS solution as previously described in section 2.2. Afterwards, the cell-laden hydrogels were cultured (37 °C and 5% CO_2) for different time periods in complete DMEM culture medium.

In Vitro Cells Encapsulation Study: Isolation of hIVD Cells and Encapsulation: Human intervertebral disc (hIVD) cells were isolated using an enzymatic digestion-based method from herniated tissue obtained from a female patient (age 36) submitted to surgery. The human biological tissue was collected in accordance to the Ethics Committee at the Centro Hospitalar Póvoa de Varzim and an informed signed consent was obtained from the patient. Briefly, the tissue was washed several times with PBS containing 1% (v/v) antibiotic-antimycotic mixture in order to completely remove blood and other bodily contaminants. The extracted tissue was separated from obvious dense annulus-like tissue and cut in small pieces in order to increase the efficiency of digestion. Human tissue digestion was performed by incubation at 37 °C in a humidified atmosphere of 5% CO_2 for 24 hours in 20 mL of cell medium Dulbecco's modified Eagle's medium:Nutrient mixture F12 1:1 (DMEM:F12; Invitrogen, USA) containing 0.3% (w/v) collagenase type II (Sigma-Aldrich Co., USA) and supplemented with 10% (v/v) FBS, 1% (v/v) of an antibiotic-antimycotic mixture containing 10 000 units·mL^{-1} penicillin G sodium, 10 000 µg·mL^{-1} streptomycin sulphate and 25 µg·mL^{-1} amphotericin B as Fungizone Antimycotic in 0.85% saline. After digestion, the cells were separated from the remaining tissue debris by using a cell strainer (100 µm). The isolated hIVD cells were expanded in DMEM:F12 (1:1) medium supplemented with 10% (v/v) FBS and 1% (v/v) of an antibiotic-antimycotic mixture in standard culture conditions until reaching confluence. The medium was changed every three days. The isolated hIVD cells were used for encapsulation within the iGG-MA and phGG-MA hydrogel discs at passage 4 (P4). Confluent cells were detached from the culture flasks using 0.25% trypsin/EDTA solution. Afterwards, a diluted cell suspension was prepared and centrifuged at 1200 rpm for 5 minutes. Prior encapsulation of hIVD cells in the hydrogels, the medium was completely aspirated and the cell pellet was re-suspended in the respective hydrogel solution, i.e., GG-MA or MBF-containing GG-MA solution. The cell-loaded hydrogels were produced in cylindrical silicone moulds with 6 mm diameter by using 2×10^5 cells per 100 µL of hydrogel. The hydrogel discs with encapsulated hIVD cells were either ionic- (in PBS solution) or photo-crosslinked (UV light at 366 nm for 6 min). Afterwards, hIVD cells/hydrogel discs were cultured in complete DMEM:F12 culture medium at 37 °C and in 5% CO_2 atmosphere for different periods of culturing.

In Vitro Cells Encapsulation Study: Calcein-AM Staining: Cell viability was assessed using calcein-AM staining, after 1, 3, 7, 14, and 21 days of culturing. Briefly, a calcein-AM (Molecular Probes, USA) solution of 1/1000 was prepared in serum-free culture medium without phenol red. The cells-laden iGG-MA and phGG-MA hydrogels were washed with PBS once, transferred to a 24-well tissue-culture polystyrene (TCPS) plate and incubated in the calcein-AM solution for 15 minutes at 37 °C and with 5% CO_2. The specimens were washed in sterile PBS and observed under fluorescence microscopy using an Axio Imager.Z1m light microscope (Zeiss, Germany) with an attached digital camera AxioCam MRm (Zeiss, Germany) connected to the AxioVision image processing software (Zeiss, Germany). All experiments were repeated three times using a minimum of two samples per experimental condition.

In Vivo Study: Subcutaneous Implantation: The protocol for the in vivo study was performed as approved by the Institutional Animal Care Committee of Padua University. Adult female Lewis rats (Charles River Laboratories), weighing 150–200 g, were subjected to the surgical procedures under halothane anesthesia and aseptic conditions. Ionic- and photo-crosslinked gellan gum hydrogels were implanted in the abdominal area. Throughout small (0.3 cm) skin incisions, the iGG-MA and phGG-MA hydrogels were implanted in a subcutaneous (s.c.) pocket above the abdominal fascia. Skin incisions were closed with nylon 5 mm stitch. No anticoagulants and prophylactic antibiotic were administered to animals before or after surgery. All surgical procedures were performed in the same way by a single surgeon. The animals were fed an unrestricted standard diet. After 10 and 18 days of implantation, the animals were sacrificed by an overdose of gaseous anesthetic, and the implants retrieved for further characterization.

In Vivo Study: Haematoxylin & Eosin (H&E) Staining: Explants were transferred to a fixative solution for 4 hours and then immersed in PBS solution with 0.02% sodium azide. Afterwards, the specimens were stored at 4 °C until the final preparation. The explants were dehydrated in graded ethanol solutions (70–99.5%), cleared in xylene, and then embedded in paraffin.[43] Sections of the explants (5 µm) were cut on a standard microtome, and routine light microscopy examination was performed after staining with H&E for evaluation of biocompatibility and cell infiltration into the implants.

In Vivo Study: Quantitative Analysis of Cells: In order to analyze the cellular response to biomaterials, masked microscopic examinations were performed by means of H&E staining for inflammatory cells.[42] Briefly, two researchers analyzed in a masked fashion at least 3 slides for each experiment by light microscopy, using 20× as the initial magnification. Each slide contained 3 sections of specimen, and 5 fields of 322 mm^2 each were analyzed for each tissue section. Experiments were performed at least three times and values are expressed as mean ± SD.

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